$CD4^+$ T cell-dependent intracellular IFN-γ production was quantitated by flow cytometry. The results, as presented in Figure 10, show an antigen-specific, IFN-γ response for splenocytes from mice immunized with base vector pcDNA3.1 or Cp protein expression constructs pWNVh-DJY or pWNVy-DJY. Splenocytes isolated from pWNVy-DJY-immunized mice, expressed higher levels of IFN-γ upon stimulation with *in vitro* translated Cp protein, than did the splenocytes isolated from pWNVh-DJY-immunized mice.

Example 4: Examination of Apoptosis by the TUNEL Assay.

Apoptosis in individual cells was determined by the TUNEL assay, in three different cell lines: HeLa cells, RD cells, and 293 cells. Cells were transfected with either the pWNVh-DJY or pWNVy-DJY construct and examined for apoptosis by the TUNEL assay. Both constructs induced apoptosis in all three cell lines.

The TUNEL assay was carried out using the *"In situ* Cell Death Detection Kit, Fluorescein" (Roche Molecular Biochemicals, Indianapolis, IN), according to the manufacturer's protocol. DNA cleavage was detected by terminal transferase (TdT) labeling of free 3'-hydroxy termini in genomic DNA with fluorescein-dUTP. Briefly, cells were fixed and permeabilized with PBS, supplemented with 0.1% Triton-X, 0.1% sodium citrate, and then the cells were incubated with "TUNEL reaction mixture," containing TdT and fluorescein-dUTP. The fluorescein-linked, incorporated dUTP was detected by fluorescence microscopy.

The data for analysis of HeLa cells, RD cells, and 293 cells transfected with either pWNVh-DJY or pWNVy-DJY construct were captured with different filters in the microscope to identify specific signals. A green signal represented incorporated fluorescein into the apoptotic cells, as revealed by a FITC filter. Images of the cells were also captured with a dual filter of FITC and rhodamine to distinguish between specific apoptotic signals and background signals. Green fluorescence under the dual filter reflected a true fluorescent signal from incorporated fluorescein-dUTP. A DAPI filter was used to reveal the nuclei of the cells, which were stained with DAPI. Not all cells were TUNEL positive. Cellular morphology was revealed when the image was captured with a DAPI filter in the light field, and showed that the nuclei of the apoptotic cells were condensed.

Similar results have been obtained with the pWNVy-DJY construct in the human neuroblastoma cell line (ATCC # CRL-2266).

Example 5: Annexin V Flow Cytometry Analysis.

HeLa cells were transfected with the enhanced green fluorescent protein (EGFP) expression vector pEGFP2-N1 (Clontech) alone, as a marker of transfection, or with pEGFP2-N1 in combination with either pWNVh-DJY or pWNVy-DJY. Two days post transfection, the cells were stained with phycoerythrin (PE)-conjugated annexin V. Stained cells were analyzed by flow cytometry. Annexin V positive cell populations were counted from the gate of EGFP-positive events, and the data were acquired using CellQuest software. Up to ten-fold induction of apoptosis over the control cells was observed by treatment with WNV-Cp (Figure 13).

Example 6: Analysis of Apoptosis-Inducing Domains of WNV Cp Protein.

Peptide WNVC-P3, as described in Example 3 and Figure 11 above, was tested for its ability to induce apoptosis in cells in culture. Peptide WNVC-P3 was incubated with SH-SY5Y neuroblastoma cells (ATCC; Manassas, VA) at a concentration of 10 μg peptide per 1 x 10^5 cells. After 24 hours, TUNEL analyses were carried out. TUNEL-positive cells were identified for cells treated with the WNVC-P3 peptide, but not for cells treated with a control peptide from prostate-specific antigen (PSA).

Example 7: Immunization with pCWNVCp Induces Antigen-Specific Humoral Immune Responses.

To investigate the levels of *in vivo* immune responses generated by the DNA vaccine, mice were immunized intramuscularly with pCWNVCp or pCDNA3 control plasmid. The quadriceps muscles of 6 to 8 weeks old female BALB/c mice (Harlan Sprague Dawley, Inc., Indianapolis, IN) were injected with 100 μg of each DNA construct of interest formulated in phosphate buffered saline (PBS) and 0.25% bupivacaine-HCl (Sigma, St. Louis, MO), at 0, 4, and 8 weeks. Prior to injection and at various time points following injection, the mice were bled retro-orbitally and the sera were collected for later analysis. The collected sera samples were analyzed for specific antibody responses against Cp peptide (WNVC-P3: TLTSAINRRSSKQKKRGGKTGI) by ELISA at 1:100, 1:400, 1:800, and 1:1600 dilution. 50 μl of WNVC-P3, diluted in PBS to a concentration of 10 μg/ml, was adsorbed onto microtiter wells overnight at 4°C, as previously described in Kim *et al.*, 1998, CD8 positive T cells controls antigen-specific immune responses through the expression of chemokines, J. Clin. Invest., 102:1112-1124, which is incorporated herein by reference. The plates were washed with

PBS-0.05% Tween-20 and blocked with 3% BSA in PBS with 0.05% Tween-20 for one hour at 37°C. Mouse antisera was diluted with 0.05% Tween-20 and incubated for one hour at 37°C, then incubated with HRP-conjugated goat anti-mouse IgG (Sigma, St. Louis, MO). The plates were washed and developed with 3'3'5'5' TMB (Sigma) buffer solution.

The pre-injection sera (collected at day 0) did not show any Cp-specific antibody response (Data not shown). The mice immunized with pCDNA3 control did not show any Cp-specific antibody response, but potent Cp-specific antibody responses were detected for mice immunized with pCWNVCp (Figure 12A). Notably, the level of Cp-specific antibody response generated by DNA immunization was more potent than that of the positive hyper-immune mouse sera obtained from ATCC (Manassas, VA).

Additionally, the subclasses of WNVCp-specific IgGs induced by the DNA vaccines were determined. It has been reported that production of IgG1 isotype is induced by Th2 type cytokines, whereas the IgG2a isotype is regulated by Th1 type cytokines (Finkelman *et al.*, 1990, Lymphokine control of in vivo immunoplobulin isotype selection, Ann. Rev. Immunol., 8:303-333, which is incorporated herein by reference). For the determination of relative levels of Cp-specific IgG subclasses, anti-murine IgG1 and IgG2a conjugated with HRP (Zymed, San Francisco, CA) were substituted for anti-murine IgG-HRP. This was followed by addition of the ABTS substrate solution (Chemicon, Temecula, CA). In each step, plates were washed 3 times with the wash buffer (PBS + 0.05% Tween-20). The plates were read on a Dynatech MR5000 plate reader with the optical density at 450 nm. As shown in Figure 12B, most of the IgG response generated from DNA immunization with pCWNVCp was of the IgG2a isotype. This strong Th1-type bias was demonstrated by both the magnitude of IgG2a and the relative ratio of IgG2a to IgG1 (Th1 to Th2).

WNVCp-specific serum antibody was determined by immunoprecipitation/Western blot analysis. WNVCp protein, translated *in vitro* without radioisotope, was immunoprecipitated with an anti-6X His (C-term) polyclonal Ab (MBL, Nagoya, Japan) and resolved on a 15 % of SDS-PAGE gel and transferred to a PDVF membrane (Millipore), which was cut into strips. Each strip was incubated with mouse immune sera from pCWNVCp or pCDNA3 immunized mice (at 1:100 dilution) and hybridized with horseradish peroxidase (HRP) conjugated anti-mouse IgG at a concentration of 1:2000. After rinsing, the strips were developed with ECL Chemiluminescent detection Kit (Amersham) (Figure 12C).